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Effects of a Soaking-Fermentation-Drying Process on the Isoflavone and γ-Aminobutyric acid Contents of Soybean

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Abstract In our study, lactic acid bacteria (LAB)-fermented whey solutions were applied in the soybean soaking process to minimize bacterial contamination and to enrich the biologically functional components of isoflavone and γ-aminobutyric acid (GABA). Among the 11 LAB tested, *Bifidobacteria infantis* and a mixed culture (*Lactobacillus acidophilus*, *Bifidobacteria lactis*, and *Streptococcus thermophilus*; ABT-3) displaying the greatest β-glucosidase activity were selected to produce improved biologically functional soybean preparations. In the soybean soaking processing (without water spraying), the LAB-cultured 10% whey solution was used to soak and to ferment the soybeans and the fermented soybeans were finally dried by heat-blowing at 55°C. The processing conditions used in this study demonstrated that the final soybean product had a reduced contamination by aerobic and coliform bacteria, compared to raw soybeans, likely due to the decrease in pH during LAB fermentation. The aglycone content of the isoflavone increased up to 44.6 mg per 100 g of dried soybean by the processing method, or approximately 8-9 times as much as their initial content. The GABA contents in the processed samples increased as the processing time of soaking-fermentation proceeded as well. The soybean sample that fermented by ABT-3 culture for 24 hr showed the greatest increase in GABA content (23.95 to 97.79 mg/100 g), probably as a result of the activity of glutamate decarboxylases (GAD) released from the soybean or produced by LAB during the soaking process.

Keywords: soybean, isoflavone, γ-aminobutyric acid, lactic acid bacteria, fermentation

Introduction

Soybeans have been consumed as a basic dietary crop in many Oriental countries. Recently, the soybean has been highly praised as a substitute for foods of animal origin in many western societies (1). Soybeans are an excellent and inexpensive source of dietary proteins, carbohydrates, vitamins, and minerals and also contain several biologically functional substances such as isoflavone, γ-aminobutyric acid (GABA), soyasaponin, trypsin inhibitor, and phytic acid (2). In 1999, the US Food and Drug Administration (FDA) approved a health claim for the role of soy protein in reducing the risk of coronary heart disease (3). The health claim states that consumption of at least 25 g soybean per day could prevent cardiovascular disease. The biological activities of the soybean gained prominence in the1980s as a result of numerous research studies. Among the biologically active components identified in the soybean, extensively studied are the isoflavones, naturally occurring heterocyclic phenols found mainly in soybeans. The structures of at least 12 isoflavone isoforms have been identified. Among them, three types of soy isoflavones exist as aglycones (genistein, daidzein, and glycitein). The core structures of these isoflavones are diverged by such functional groups as malonyl- and acetyl-substitutes on β-glucoside. The total content of isoflavones in a soybean crop may vary between 1.2 to 4.2 mg/g, depending on the cultivar, the crop year and growth location (4). More than 70% of the soy isoflavone exists as the malonyl-substituted form of β-glucoside (5, 6). In terms of bioavailability, the aglycone of isoflavone is more efficiently taken into the body than is the glucoside form. The aglycones are structurally similar to the estrogens and, therefore, mimic the functions of estradiol (7). Aglycone-type isoflavones are known to be associated with the prevention and potential treatment of hormone-dependent disorders based upon epidemiological studies and some small-scale human clinical experiments (8, 9). In order to increase the content of soy aglycones, germination, fermentation, and β-glucosidase treatments have been utilized (10, 11).

GABA ($C_4H_9NO_2$) is an amino acid not normally present as a building block of proteins and is produced *in vivo* primarily by decarboxylation of L-glutamic acid via glutamate decarboxylase (GAD). GABA has several physiological functions including neurotransmission and induction of hypotension, diuresis, and as a tranquilizer (12, 13). GABA is present in a variety of plants including cereals, legumes, fruits, vegetables, mushrooms, and seaweed. Because plant GABA content is relatively low, germination, anaerobic incubation, CO_2 treatment, and fermentation with microorganisms have been utilized to increase GABA concentration in tea, brown rice, and legumes (4, 14, 15)

Soybeans have been soaked prior to food processing for 12-24 hr, which led to a significant loss of soluble solids and increased exposure to bacterial contamination. These are critical problems affecting the processing of soybean into a food. Thus, newer modifications of the soaking or substitution steps of the process should be considered in the development of safer and biologically functional soybean-based foods (16).

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In the present study, a lactic acid bacteria (LAB)-fermented whey solution was added during the soybean soaking process to minimize bacterial contamination and to likely enrich the biologically functional components of isoflavone and GABA in the soybean. We also investigated the effect of the drying process on changes in the aglycone (isoflavone) content of the soybean.

Materials and Methods

Materials Raw soybean cultivar (Suwon 97) was harvested and purchased (fall 2004) at Inje, Gangwon, Korea. Anomalously shaped and discolored soybean grains were discarded, and the remaining beans were washed five times for further experiments. Eleven types of lactic acid bacterial cultures were used for the fermentation process; 10 lactic acid bacteria, *Bifidobacteria infantis* (Maeil Dairy Industry, Ltd., Korea), *Streptococcus thermophilus, Lactobacillus rhamnosus, Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus paracasei, Lactobacillus fermentum, Lactococcus lactis, Pediococcus acetilactic* (Chr. Hansen, Denmark), *Bifidobacteria longum* (Morinaga, Japan), and one mixed culture (ABT-3) consisting of *L. acidophilus*, *Bifidobacteria lactis*, and *S. thermophilus* (Chr. Hansen, Denmark). We used 10 isoflavone standards (malonyl genistin, acetyl genistin, acetyl daidzin, acetyl glycitin, genistin, daidzin, glycitin, genistein, daidzein, and glycitein) purchased from LC Laboratories Co. (Division of PKC Pharmaceuticals, Inc., Woburn, MA, USA), and other two isoflavones (malonyl daidzin, malonyl glycitin) that were obtained from the Wako Chemical Co. (Osaka, Japan). The GABA (γ-aminoutyric acid) standard was obtained from the Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

Measurement of β-glucosidase activity The bacterial strains were activated first by growing in modified MRS broth (supplement: 0.5 g/L of L-cystein-HCl, 0.2 g/L of $NaCO_3$, and 0.1 g/L of $CaCl_2$, Difco Labories, Detroit, MI, USA) with incubation carried out at 37°C for 18-24 hr. The cells were harvested by centrifugation (10,000×*g*) at 4 °C for 5 min, washed twice with ice-cold 50 mM sodium phosphate buffer (pH 6.0) and resuspended in the same buffer. The suspended cells were disrupted by sonication on ice for 1 min. The cellular debris was removed by centrifugation (17,000×g) at 4°C for 30 min and the supernatant was used as a crude enzyme extract. The β-glucosidase activity in the crude extract was assayed by determining the rate of hydrolysis of *p*-NPG (17). The enzyme activity was determined by incubating mixtures of 10 mM of substrate (*p*-NPG) in a 50 mM sodium phosphate buffer (pH 7.0) and 50 μL of an enzyme solution at 45°C for 10 min. The reaction was stopped by adding 0.4 mL of 0.5 M sodium carbonate solution. The amount of *p*-nitrophenol released in the supernatant was measured at 414 nm. One unit of enzyme was defined as the amount of enzyme that released 1 μmol of *p*-nitrophenol from the substrate per one min.

Sample preparation by soaking and lactic acid bacteria fermentation In the preparation of the LAB-fermented whey cultures, a milk whey solution (10%, w/v) was inoculated with 1.0×10^6 CFU/mL of the selected LAB and the mixture was incubated at 40°C for 24 hr. At the end of incubation, 5 kg of pre-washed raw soybeans were soaked in 6.5 L of the fermented whey and incubated at 40°C for another 12-24 hr, with constant humidity (95% RH). Soaked and fermented soybean samples were washed three times and dried at 55°C for 24 hr using a drying oven until the sample moisture content was less than 10%. The soaked and fermented soybean samples were washed three more times and lyophilized for further analysis.

Measurement of the pH, titratable acidity, and count of viable cells Thirty g of soybean sample in 270 mL of sterilized distilled water was homogenized using a homogenizer (PT MR-3000; Kinematica, Switzerland) to determine pH and titratable acidity (TA). The pH was measured using a pH meter (Orion Research Inc., Beverly, MA, USA), and the TA was determined by the AOAC method 942.15 (18) and expressed as % lactic acid. To enumerate the general, coliform, and lactic acid bacteria, plate count agar (Difco), EMB agar (Difco), and BL agar (Difco) were used, respectively. When the bacterial cell counting was performed, a serially diluted sample (1 mL each) was pour-plated onto the appropriate medium. After 48 hr of incubation at 37°C, the colonies that appeared on the plates were counted and the CFU per mL was calculated.

Extraction and quantitative analysis of the isoflavones Analysis of the isoflavones was performed following the method of Wang and Murphy (5) with slight modification. For the extraction of the isoflavones, 0.5 g of ground sample in 10 mL of 70% methanol was vigorously shaken and extracted at room temperature for 30 min using an ultrasonicator (Bransonic, Danbury, CT, USA). The extract was centrifuged at 12,000×g for 15 min and the supernatant filtered through a syringe filter (0.22 μm, Waters Co., Milford, MA, USA) prior to HPLC (1100 series; Agilent, Santa Clara, CA, USA) analysis. The HPLC analysis condition for the isoflavones is described in Table 1.

Extraction and quantitative analysis of GABA For the extraction of GABA, 0.5 g of ground sample in 10 mL of 10% sulfosalicylic acid was vigorously shaken and extracted at room temperature for 30 min using an ultrasonicator (Bransonic). The extract was centrifuged at 12,000×g for 15 min and the supernatant then filtered through a syringe filter (0.22 μm, Waters Co.) and derivatized using *o*-phthalic aldehyde (OPA) prior to HPLC (1100 series; Agilent) analysis. The HPLC analysis conditions for GABA are shown in Table 1.

Results and Discussion

β-Glucosidase activity of lactic acid bacteria The activities of β-glucosidase produced by the LAB cultures were determined after 18-24 hr of incubation at 37°C (Fig. 1). The β-glucosidase activity measured from the cultures of *B. infantis* and the ABT-3 was at the levels of 7,900 and 4,479 μmol/min, respectively, while other LAB displayed no more than 2-10 μmol/min. Therefore, the *B. infantis*

Table 1. Instrument and operating conditions for HPLC analyses of isoflavones and GABA

	Isoflavone	GABA
HPLC system	Agilent 1100 series	
Column	Eclipse XDB-C18 (4.6 cm × 150 mm, 3.6 um)	
Guard column	Eclipse XDB-C18	
Eluents (gradient)	A: 0.1% Phosphoric acid B: Acetonitrile	A: 40 mM Na_2HPO_4 (pH 7.8) B: Acetonitrile/Methanol/H_2O (45/45/10, v/v)
Flow rate	0.8 mL/min	2.0 mL/min
UV detector	Agilent 1200 series multiple wavelength detector	
Detector wavelength	254 nm	338 nm

and ABT-3 preparations were selected to catalyze the hydrolysis of the soybean glycoside isoflavone during the soaking-fermentation period and additional analyses.

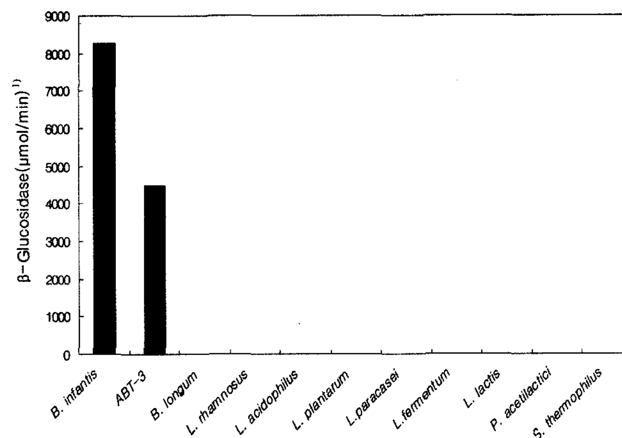

Fig. 1. β-Glucosidase activity of the selected lactic acid bacterial species. [1)]The enzyme activity is expressed by the released amount of *p*-nitrophenol from *p*-nitrophenyl glucoside per unit time.

Changes in pH, TA, and viable cell counts The changes in pH, TA, and viable cell counts of the processed soybeans were measured after LAB fermentation and drying procedures (Table 2). As the soaking period progressed, pH decreased and TA increased in the soaked soybeans. The pH decreased from 6.4 to 5.1, while the TA increased from 0.23 to 0.38% after 24 hr of soybean fermentation with both the *B. infantis* and ABT-3 cultures. When the selected LAB were inoculated in 10% whey solution, viable cell counts of the ABT-3 increased from 1.0×10^6 to 1.7×10^8 CFU/g during the 24 hr incubation whereas with *B. infantis,* the viable cell count remained at the initial inoculation level (1.0×10^6 CFU/g) indicating no additional growth. During the soybean soaking with LAB-fermented whey for up to 24 hr, both *B. infantis* and ABT-3 cell counts finally reached a level of more than 1.0×10^8 CFU/g. Thus, this observation indicated that *B. infantis* had a longer lag phase than did ABT-3 under this incubation condition.

When the number of aerobic and coliform bacteria of the fermented soybeans was compared to that of the raw soybean, substantial decreases in these bacterial species were observed from all soaking treatment groups; aerobic bacterial counts of D, E, and F sample groups, however, increased after the drying process by heat-blowing at 55°C, presumably because the antibacterial effect of *B. infantis* against thermostable strains might be weaker than that of ABT-3. These results agreed with the previously

Table 2. Changes of pH, titratable acidity, TA, and bacterial cell counts in soybeans soaked and fermented with lactic acid bacteria at 40°C after drying at 55°C

		Culture / Drying	time (hr)	pH	TA (%)	Aerobic bacteria	Coliform bacteria	Lactic acid bacteria
Raw soybean		-	-	6.4	0.23	7.3×10^4	2.0×10^2	1.8×10^4
ABT-3[1)]	Control[2)]	Culture	0	5.9	0.24	1.0×10^3	1.0×10	1.7×10^8
	A	Culture	12	5.4	0.31	2.0×10^2	2.0×10	1.6×10^8
		Drying	24	5.2	0.34	1.0×10^2	1.0×10	5.1×10^5
	B	Culture	18	5.0	0.35	2.5×10^3	1.0×10	1.5×10^8
		Drying	24	5.0	0.33	2.0×10^3	1.0×10	1.5×10^4
	C	Culture	24	5.1	0.38	2.7×10^4	1.0×10	4.8×10^7
		Drying	24	5.0	0.38	2.5×10^2	1.0×10	1.2×10^3
B. infantis	Control	Culture	0	5.9	0.19	1.0×10^3	2.0×10	6.5×10^5
	D	Culture	12	5.5	0.16	5.7×10^3	1.0×10	3.9×10^8
		Drying	24	5.4	0.32	2.5×10^4	1.0×10	7.0×10^4
	E	Culture	18	5.3	0.36	3.8×10^4	1.0×10	3.6×10^8
		Drying	24	5.2	0.34	5.0×10^4	1.0×10	7.4×10^3
	F	Culture	24	5.1	0.38	5.0×10^4	1.0×10	6.5×10^8
		Drying	24	4.9	0.35	5.0×10^4	1.0×10	1.0×10^2

[1)]ABT-3 was a mixed culture of *L. acidophilus, B. lactis, S. thermophilus.*
[2)]Control: soybean + lactic acid cultured media.

reported findings (19, 20) that *L. acidophilus* and *bulgaricus* had antibacterial activities against coliform bacteria. Especially a mixed culture consisting of *L. acidophilus*, *B. lactis*, and two other LAB strains had significantly lower incidence of potentially pathogenic bacteria (21).

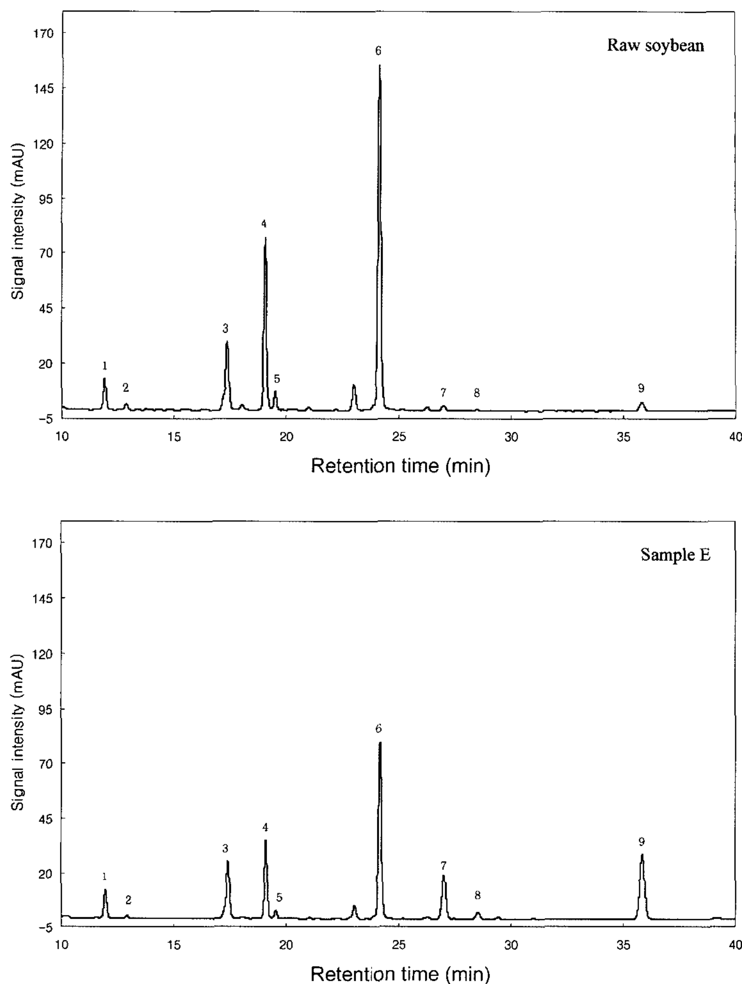

Fig. 2. Chromatographic separation of isoflavones extracted from raw soybean and samples E. 1, Daidzin; 2, glycitin; 3, genistin; 4, 6"-o-malonyldaidzin; 5, 6"-o-malonylglycitin; 6, 6"-o-malonylgenistin; 7, daidzein; 8, glycitein; 9, genistein. Sample E was a 24-hr dried soybean preparation after 18 hr of soaking and fermentation with *B. Infantis*.

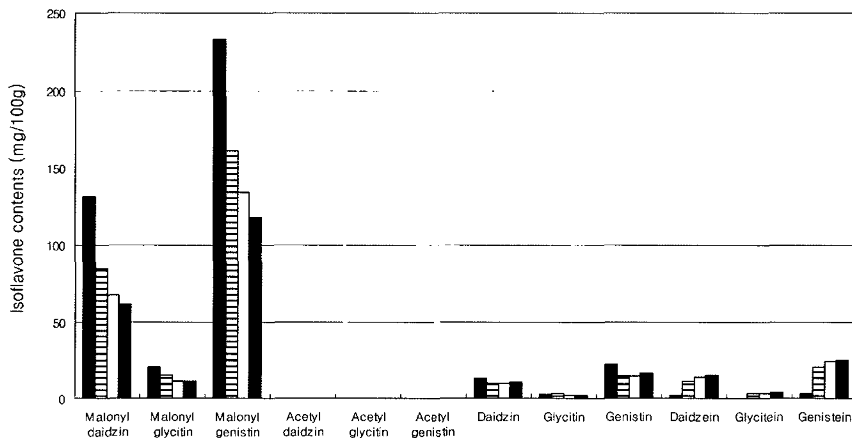

Fig. 3. Changes in isoflavone contents in dried soybeans after soaking and fermentation with the ABT-3 culture. ABT-3 was a mixed culture of *L. acidophilus*, *B. lactis*, and *S. thermophilus*. The symbols represent 24 hr dried soybean after 0 (■), 12 (▤), 18 (□), and 24 hr (▦) soaking and fermentation with the ABT-3 culture.

Changes in isoflavone contents during the fermentation-soaking process Quantitative analysis of the isoflavone content during the fermentation-soaking process was achieved by the HPLC method (Table 1). The HPLC profiles of the isoflavones extracted from raw and processed soybeans (sample E) are represented in Fig. 2, with the isoflavone content of raw soybean shown in Table 3. The raw soybean contained a total of 474.73 mg of isoflavone/100 g of soybean, with the malonyl-substituted isoforms of isoflavone contributing the greatest amount (402.16 mg/100 g, corresponding to 84.7% of total quantifiable isoflavones, Table 3). The contents of each isoflavone isoform in the soybeans were measured as follows: malonyl-substitutes > glucoside types > aglycone types > acetyl-substitutes. This pattern of results agreed reasonably well with previous studies (5), even though the isoflavone content of the soybeans varied depending on such factors as cultivar, growth location, harvesting year, and possible other environmental influences (4). Once the raw soybeans were processed by fermentation and drying, the isoflavone content was shown to be affected by the LAB strains used as well as the processing time (Fig. 3 and 4). Total isoflavone contents decreased about 41% (from 474.75 to 276.17 and 279.92 mg/100 g in soybeans fermented with *B. Infantis* and ABT-3, respectively). The content of the two glucosides daidzin and glycitin did not change significantly but that of the remaining glucoside, genistin, substantially decreased from 26.94 to 11.6 mg/100 g. The content of malonyl-glucosides decreased nearly 50% (from 385.27 to 190.71 mg/100 g) as well. On the other hand, the content of aglycone types increased from 5.4 to 35.13, 41.53, and 44.62 mg/100 g in samples A, B, and C (about 8. 3 fold); and from 4.85 to 31.97, 40.13, and 37.58 mg/100 g in D, E, and F samples, respectively. In addition, a slightly greater increase in the aglycone-type isoflavone was observed from the fermented soybeans with *B. Infantis*, compared to those with ABT-3. Such an increase in aglycone content observed from the fermented soybeans (approximately 8-9 folds) was much greater than the 4-5 fold increase reported previously (10). It has been suggested that the β-glucosidase derived from LAB converted the glycosides to aglycones (22). An overall significant increase in aglycones was observed even though there was a substantial loss of total isoflavone content during the soaking-fermentation process.

Changes in GABA content during the fermentation-soaking process The GABA content was also monitored by HPLC analysis during the processing. The HPLC separation of the extracted GABA from raw and processed soybeans (sample C) is presented in Fig 5. The GABA contents in the processed samples were shown to increase with the duration of the processing soaking-fermentation time (Table 4). The initial GABA content of the raw soybean was 25.72 mg/100 g. The sample C that was fermented by the ABT-3 culture for 24 hr showed the greatest increase in GABA content (from 23.95 to 97.79 mg/100 g), which was followed in GABA content by samples F, B, A, E, and D. It is not certain whether the LAB strain types affected the amount of GABA produced during the process. The increase in GABA content in our study was comparable to the previously reported result that GABA content of fermented soymilk increased 3.85 fold from 93.9 to 361.6 mg/100 g (23) and it has been

Table 3. Isoflavone contents of raw soybean determined by HPLC analysis (mg/100 g)

Sample	Isoflavone[1]												
	MGI	MDI	MGYI	AGI	ADI	AGYI	GI	DI	GYI	GE	DE	GY	TI
Raw soybean	248.71	133.79	19.66	ND[2]	ND	ND	26.94	15.40	3.24	3.79	2.34	ND	474.73

[1]MGI, malonyl genistin; MDI, malonyl daidzin; MGYI, malonyl glycitin; AGI, acetyl genistin; ADI, acetyl daidzin; AGYI, acetyl glycitin; GI, genistin; DI, daidzin; GYI, glycitin; GE, genistein; DE, daidzein; GY, glycitein; TI, total isoflavone content.
[2]ND: Not detectable.

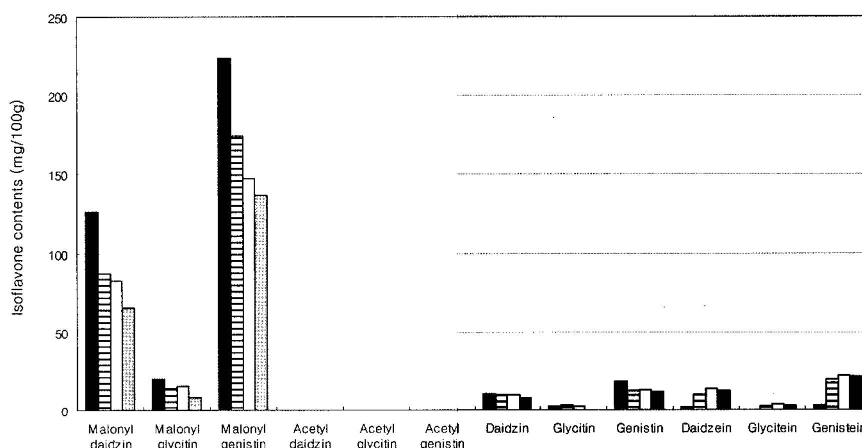

Fig. 4. Changes in isoflavone contents in dried soybeans after soaking and fermentation with *B. infantis*. The symbols represent the 24 hr dried soybean after 0 (■), 12 (▤), 18 (□), and 24 hr (▦) soaking and fermentation with *B. infantis*.

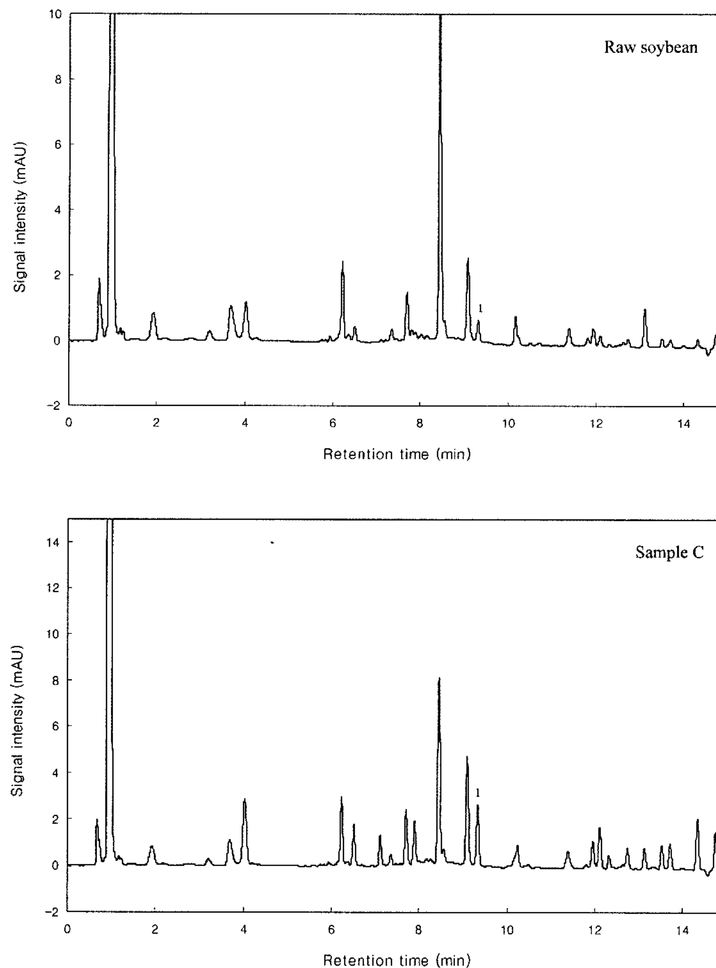

Fig. 5. Chromatographic separation of GABA extracted from raw soybean and sample C. Peak 1 in the chromatogram was identified as GABA; sample C was a 24 hr dried soybean preparation after a 24 hr soaking period and fermentation with the ABT-3 culture.

suggested that the glutamic acid in the soybean and whey was effectively transformed to GABA by the glutamate decarboxylase (GAD) released from the soybean itself and produced by LAB during the soaking process.

Table 4. Changes in the GABA content of soybeans soaked and fermented with lactic acid bacteria at 40°C after drying at 55°C

		Culture (hr)	Drying (hr)	GABA (mg/100 g)
Raw soybean		-	-	25.72
ABT-3[1]	Control[2]	-	-	23.95
	A	12	24	84.09
	B	18	24	90.65
	C	24	24	97.79
B. Infantis	Control	-	-	23.37
	D	12	24	55.61
	E	18	24	65.45
	F	24	24	91.45

[1]ABT-3 was a mixed culture of *L. acidophilus, B. lactis, S. thermophilus.*
[2]Control: soybean + lactic acid cultured media.

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